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A cation study on rice husk biomass pre-treatment with aqueous hydroxides: Cellulose solubility does not correlate with improved enzymatic hydrolysis

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**A cation study on rice husk biomass pre-treatment with
aqueous hydroxides: Cellulose solubility does not correlate
with improved enzymatic hydrolysis**

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Abstract

Biomass pre-treatment is a key first step in converting recalcitrant lignocellulosic biomass into value-added products. Aqueous hydroxide solutions can be effective biomass pre-treatment media, and the cation of the hydroxide salt can have an extremely significant effect upon the physicochemical behaviour of the hydroxide solution. However, the cation effect has not been comprehensively investigated with respect to biomass pre-treatment. Here we have investigated pre-treatment of rice husks (from *Oryza sativa*) and shown that the cation indeed has a significant effect upon downstream enzymatic hydrolysis of the cellulose (with cellulase). In particular, the ability of the solution to dissolve cellulose was negatively correlated with pre-treatment effectiveness, as judged by the downstream glucose yield. This was observed by investigating aqueous solutions of lithium, potassium, caesium, tetramethylammonium, tetraethylammonium, tetrapropylammonium, tetrabutylammonium and tetrahexylammonium hydroxide. Silica solubility was almost cation-independent, lignin solubility was moderately cation-dependent, while cellulose solubility was strongly cation-dependent. The rate of lignin extraction was inversely correlated with the size of the cation. As cellulose-dissolution is a demanding chemical process, it initially limited the ability of the solution to disrupt the whole biomass, necessitated extensive washing of the pre-treated rice husk, and still resulted in significant cation contamination downstream. Overall, lithium hydroxide was found to be the most effective hydroxide.

Keywords: Rice hulls, onium hydroxides, cellulase, kinetics

Introduction

Lignocellulosic biomass represents a non-edible, sustainable supply of chemical matter which is produced at a rate of *ca.* 200 billion tons a year¹, and is primarily composed of cellulose, hemicellulose and lignin, with differing ratios depending on biomass type. Rice husks (or hulls) are an example of lignocellulosic biomass that is also a major agricultural waste product.² It comprises *ca.* 20% of dry weight in harvested rice, and is an especially resistant biomass as it contains 15-24 wt% silica.³⁻⁵ This makes it chemically resistant (especially to acids⁵), biochemically resistant⁶ and destructive to mechanical equipment.⁴ For these reasons it cannot be easily digested by animals^{2,6} and is typically buried as low-density landfill or burnt.⁷

One goal of lignocellulosic biomass pre-treatment is to overcome the lignin-hemicellulose barrier to increase enzyme accessibility to the cellulose,⁸⁻⁹ and in the case of rice husks, also to break down the silica shell. A solution that could disrupt the rice husk structure is essential for separation and further utilisation of its components. Strongly alkaline solutions (containing the hydroxide anion) can disrupt the inter- and intra-lignocellulosic hydrogen bonds present in the biomass,¹⁰ resulting in swelling and potentially even dissolution of the biomass. Hydroxide solutions are also known solvents for silica¹¹ and have been found to be effective in disrupting the silica layer surrounding rice husks.⁴⁻⁵

Alkali metal hydroxides such as sodium hydroxide (Na[OH]) are widely employed for the swelling of cellulose.¹²⁻¹⁴ Solutions of Na[OH] can dissolve cellulose, but only below 4°C.¹⁵⁻¹⁶ Conversely, several tetraalkylammonium (and much more recently, tetraalkylphosphonium, [P₄₄₄₄]⁺) hydroxide solutions are widely known to dissolve cellulose

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3 at room temperature.¹⁷⁻²¹ Dissolution with tetrabutylammonium hydroxide has been
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5 demonstrated to occur down to a molecular level, whereas 'solutions' of cellulose in Na[OH]
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7 still contain aggregates of cellulose chains.^{17, 21} Tetramethylammonium hydroxide
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9 ([N₁₁₁₁][OH]), while unable to dissolve cellulose, has been reported to be a far superior
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11 cellulose swelling agent compared to Na[OH].²²⁻²³ The superior performance of
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13 tetraalkylammonium hydroxide solutions for both cellulose swelling and dissolution is likely
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15 related to amphiphilic and hydrophobic interactions between the cellulose and the cation.²⁴⁻
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Unfortunately, comparative studies of lignocellulosic biomass processing with
different cations are relatively rare, despite the expected difference in interactions with
lignocellulosic components. Rice husks and straw have been pre-treated in various studies
prior to down-stream enzymatic processes using just one cation, *e.g.* Na[OH],^{26-28,29}
Na[OH]/H₂O₂,³⁰ Ca[OH]₂,³¹ and choline hydroxide.³² For cation comparisons, the pre-
treatment of rice straw was evaluated using Na[OH] (at 55°C) and Ca[OH]₂ (at 95°C), with
the Ca[OH]₂ resulting in higher down-stream saccharification yields.³³ Limited comparisons
of Na[OH] and K[OH] have also been performed on rice-based agricultural waste; pre-
treatment with K[OH] was more effective for downstream production of cellulase via
fermentation,³⁴ whereas Na[OH] was more effective as judged by downstream sugar
production by cellulase³⁵ (both pre-treatment studies performed at 121°C). Rice husk pre-
treatment with [P₄₄₄₄][OH] (at room temperature) followed by acid or enzymatic hydrolysis
has been investigated, and compared to straight acid treatment and rice husks pre-treated
in refluxing K[OH].⁵ Pre-treatment of switchgrass with [N₄₄₄₄][OH] (at 50°C) then
downstream enzymatic saccharification has also been reported.³⁶ From these various

studies, performed with very different conditions, no meaningful trend can be extrapolated beyond the fact that cations can clearly have an effect.

We have therefore compared the performance of aqueous hydroxide solutions as biomass pre-treatment media (at room temperature), while systematically varying the cation. The hydroxide cation was lithium, potassium, caesium or a tetraalkylammonium cation, where alkyl = methyl, ethyl, propyl, butyl or hexyl; all alkyl chains were the linear (n-) isomer. Whole rice husks were pre-treated, with the effectiveness judged by downstream enzymatic hydrolysis with cellulase.

Experimental

Cellulose solubility in hydroxide solutions

Tetraalkylammonium hydroxide solutions with the desired $\text{H}_2\text{O}:[\text{OH}]^-$ molar ratios (180:1, 90:1, 45:1, 22.5:1 or 11.25:1) were prepared by diluting a commercial stock solution or reducing water content by evaporation at 80°C in a Teflon beaker. Please note that $[\text{N}_{1111}][\text{OH}]$ is highly toxic.³⁷ Alkali metal hydroxides were prepared by direct dissolution of the solid hydroxide salt. Avicell cellulose (Sigma Aldrich, Castle Hill) was added in 0.25 wt% proportions and stirred at ambient temperature ($20 \pm 2^\circ\text{C}$) at 400 rpm. The cellulose either dissolved to form a clear solution (dissolution confirmed by microscope evaluation), or remained undissolved. If cellulose remained undissolved after 24 hours of stirring, or the solution became too viscous to be stirred (*e.g.* a gel), the solution was considered saturated.

Cellulose solubility in aqueous tetraalkylammonium hydroxide solutions has been reported to be temperature-dependent,¹⁹ and sensitive to alkali metal contaminants.¹⁸ All experiments were performed at ambient temperature in a temperature-controlled laboratory ($20 \pm 2^\circ\text{C}$), unless otherwise noted. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) was used to confirm low contents of lithium ($<0.1 \text{ mg L}^{-1}$), sodium ($<1.5 \text{ mg L}^{-1}$) and potassium ($<0.35 \text{ mg L}^{-1}$) in the commercial tetraalkylammonium hydroxide solutions (when the water: $[\text{OH}]^-$ molar ratio was 22.5:1). Multiple bottles were ordered, and any bottle that exceeded these values (typically values were either low or $>50 \text{ mg L}^{-1}$) was not used in this study.

Silica solubility

Silica gel (40-63 μm , Grace Davison Discovery Sciences) solubility was determined exactly as reported elsewhere.⁵

Lignin solubility

Alkaline lignin (Tokyo Chemical Industry Co., Ltd) was added to 5 mL hydroxide solutions of the desired $\text{H}_2\text{O}:[\text{OH}]^-$ ratio, where it rapidly dissolved to form a black solution. The addition of lignin was continued until either the solution became too viscous to be stirred (at ambient temperature), or undissolved powder remained after 24 h (confirmed optically using a microscope).

Pretreatment of rice husks and downstream handling

The rice husks were pretreated (as described below) and then isolated and either analysed or subjected to enzymatic hydrolysis. Whole rice husks (SunRice, Australia, from *Oryza sativa*) were used as received, with no prior drying, washing, grinding or sieving. The air-equilibrated rice husks contained *ca.* 10 wt% water.

Pre-treatment was carried out using 2 wt% rice husk, typically 0.1 g of rice husk in 5 mL hydroxide solution. The *ca.* 8 mm long by 2 mm wide rice husks were stirred in the relevant solutions for 72 h at room temperature.

Prior to enzymatic hydrolysis, 50 mL of anti-solvent (either water or methanol, as noted in the text) was added to precipitate dissolved materials such as cellulose. This mixture was stirred for 20 mins, then filtered through a 10 μ m nylon Millipore filter. The solid residue obtained were either (i) taken immediately for enzymatic hydrolysis, or (ii) dried, weighed and analysed for lignin and silica content.

The effect of the different cations and the $\text{H}_2\text{O}:[\text{OH}]^-$ ratio upon the bulk structure of the rice husk was also evaluated. After stirring in the hydroxide solution, the mixture filtered through a ceramic filter (pore diameter 0.56 mm) to recover “bulk residue” (material still >0.56 mm). The amount of remaining solid residue larger than 0.56 mm was used to evaluate the extent of bulk rice husk structure disruption. The filtrate was centrifuged (5000 rpm, 1 min) to separate all remaining solid residue (<<0.56 mm) from the dissolved material, which was identified as “digested residue”.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed using methodology adapted from the NREL recommended guidelines,³⁸ as reported extensively elsewhere.^{5, 39} Each measurement was done in triplicate.

Effect of residual cations upon enzymatic hydrolysis of pure cellulose

In order to investigate the effect of different cations upon enzymatic hydrolysis in acetate buffers, 50 mM buffers were prepared using 20 mM acetic acid and 30 mM of the relevant acetate salt. Where the acetate salt was not available, a solution containing 30 mM of the relevant hydroxide and 50 mM acetic acid was prepared. The pH of the resulting buffers all fell within the range of 4.81 to 4.93. To this, 33 mg of cellulose (finely cut Whatman filter paper no. 1) was added to 5 mL of the buffer and shaken at 50°C for 48 h.

Kinetics of lignin extraction

To measure the kinetics of lignin extraction from rice husks, hydroxide solutions with a $\text{H}_2\text{O}:[\text{OH}]^-$ ratio of 45:1 were prepared. The UV-Vis spectra of 3 mL of these solutions were recorded as background spectra in 10 mm path length quartz cuvettes. Then 7.5 mg of rice husks (*ca.* 3 rice husks) were added to this solution and stirred at 1200 rpm. The spectra were recorded over a 24 h period to monitor how much lignin was extracted.

Acid hydrolysis for lignin content determination

Acid hydrolysis was performed following guidelines suggested by NREL,⁴⁰ while using previously reported modifications for the high ash content common to rice husks.⁵

Thermogravimetric analysis (TGA) for ash content determination

All thermogravimetric (TGA) analysis to determine the silica-rich ash content was performed as reported elsewhere.⁵

Results and discussion

Interaction of hydroxide solutions with models for the three major constituents of rice husk, as a function of cation

The composition of the rice husks used in this study were recently investigated and found to contain 9.8 wt% water, and (on a dry weight basis) be composed of 19 (± 2) wt% lignin, 20 (± 2) wt% silica and 62 (± 6) wt% holocellulose.⁵ Prior to investigating the whole biomass, the solubility of three model compounds, alkali lignin, silica gel and microcrystalline cellulose, was quantified for all eight cations at a fixed ratio of [cation][OH] $\cdot 22.5\text{H}_2\text{O}$. The observed solubility values are summarised quantitatively in Figure 1. Briefly, (i) all systems dissolved *ca.* 14 ± 1 wt% silica, and silica solubility was cation-independent; (ii) alkali lignin was highly soluble in all systems with the exception of $[\text{N}_{666}][\text{OH}]$; and (iii) cellulose solubility displayed a ‘Goldilocks’ zone where if the cation was neither too big nor too small, cellulose could be dissolved.

Silica dissolution likely proceeds by chemical reaction exclusively with the hydroxide anion, followed by solubilisation of the $[\text{Si}(\text{OH})_4]^-$ anion.^{5, 41} These results demonstrate that silica dissolution and solvation is essentially independent of the nature of the cation (within uncertainty), all systems dissolving $ca. 14 \pm 1$ wt% amorphous silica gel.

The influence of the $[\text{cation}][\text{OH}]\cdot\text{H}_2\text{O}$ ratio upon silica solubility was also investigated, and is displayed graphically in Figure S1 in the Electronic Supplementary Information (ESI) section. Silica solubility increased as the amount of hydroxide increased, reaching a maximum of 15 wt% silica for a $\text{H}_2\text{O}:[\text{cation}][\text{OH}]$ ratio of 22.5:1. Silica solubility decreased beyond this ratio, likely due to a lack of water to effectively hydrate and solubilise the silicate anion.

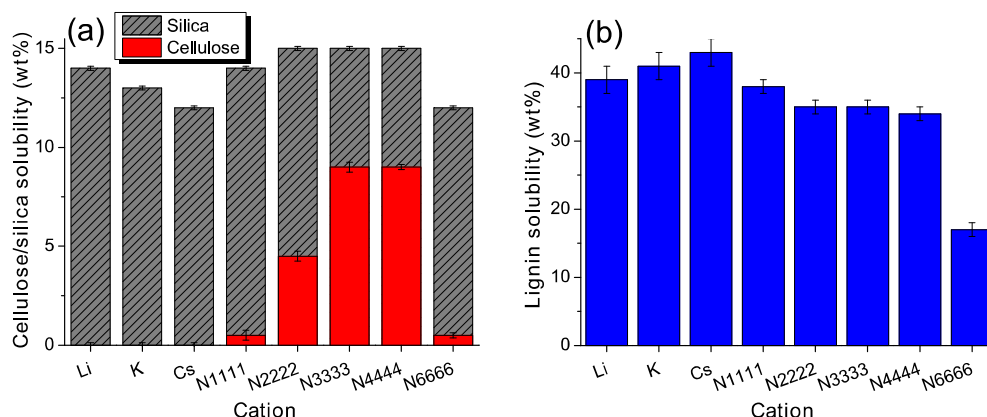


Figure 1: Solubility of the major rice husk components (cellulose and silica in (a); lignin in (b)) in a range of hydroxides ($\text{H}_2\text{O} : [\text{OH}] = 22.5 : 1$), highlighting excellent lignin solubility (with a minor cation effect), high silica solubility (with a very minor cation effect) and that a limited range of cations also display good cellulose solubility. Error bars for cellulose and lignin solubility express half the range between clearly soluble and clearly insoluble/gelled systems; the silica solubility error bars are from triplicate ICP-OES determinations.

Alkali lignin was used as a model lignin compound. Although it is widely used as a lignin analogue⁴² it also demonstrates moderate water solubility (unlike genuine lignin) hence it can only be used as a qualitative rather than quantitative model. Solubility was confirmed by optical inspection using a microscope, and the solubility limit was typically reached when the entire sample turned solid or gelatinous, with the exception of [N₆₆₆₆][OH] which had a clear solubility limit, with undissolved lignin being present as a powder rather than the entire system forming a gel. Lignin was observed to be highly soluble in all alkali samples, likely due to deprotonation of phenolic groups to form water-soluble phenolates, and only the largest cation, [N₆₆₆₆]⁺, displayed a significant cation effect. Studies of various alkyl chain lengths on cations of ionic liquids, and their effect upon the bulk structure, have noted clear evolution of continuous non-polar (alkyl chain-rich) domains for hexyl groups or longer.⁴³ Therefore the [N₆₆₆₆]⁺ is likely excessively hydrophobic, and increased cation-cation interactions could have resulted in reduced lignin solubility.

Commercial microcrystalline cellulose powder (Avicell) was used as the cellulose model compound. This is extremely widely employed as a model compound for lignocellulosic cellulose.⁴⁴ However, it should be noted that the average degree of polymerisation in Avicell (*ca.* 200) is much shorter than cellulose found in lignocellulosic biomass (*ca.* 1,000 – 5,000),⁴⁵ which could have implications regarding dissolution kinetics, overall solubility and the physicochemical properties of the resulting solution. As shown in Figure 1, cellulose was found to be insoluble at 20°C from Li[OH] through to Cs[OH]. Cellulose was highly soluble in [N₃₃₃₃][OH] and [N₄₄₄₄][OH], moderately soluble in [N₂₂₂₂][OH] and sparingly soluble in [N₁₁₁₁][OH] and [N₆₆₆₆][OH]. Study of [N₅₅₅₅][OH] is not reported here,

as we were unable to source this solution with a low enough Na⁺ and K⁺ content for meaningful comparison (*c.f.* reference¹⁸).

The cellulose solubility trends observed generally agree well with prior literature. For example, our observation that cellulose is slightly soluble in [N₁₁₁₁][OH] (up to 0.75 ± 0.25 wt% dissolving) falls between several reports that note none or partial cellulose dissolution^{22, 46-48} and Zhong *et al.* who recently noted high cellulose solubility.⁴⁹ Our observation that [N₂₂₂₂][OH] could dissolve up to 4.5 wt% cellulose also agrees with several reports,^{18, 50} although Abe *et al.* reported no cellulose solubility.²⁰ Highest solubility was observed in [N₃₃₃₃][OH]; no other reports could be found except Lieser and Leckzyck's report in 1936 that solutions above 1.8 M [N₃₃₃₃][OH] in water can dissolve cellulose.⁴⁸ Cellulose was highly soluble in [N₄₄₄₄][OH] (consistent with prior reports^{17, 19, 21, 49, 51-53}) although gelation occurred before true saturation could be achieved. The most 'oily' cation, [N₆₆₆₆][OH], displayed only minor cellulose solubility (at the ratio of 22.5:1), in agreement with the observations of Abe *et al.*²⁰ The deviations between our work and a minority of other studies could be related to the high sensitivity of cellulose dissolution to contamination by alkali metal cations¹⁸ and even the ambient temperature (lower temperatures being preferential¹⁹). The temperature and alkali metal contents for our systems have therefore been included in the Experimental section.

Cellulose solubility was also evaluated as a function of the H₂O:[cation][OH] ratio (Figure S2). Significant cellulose solubility was only observed for a ratio of 22.5:1 or lower; solubility increased at 11.25:1, but lower ratios could not be achieved due to decomposition of the tetraalkylammonium cations.^{5, 20} Overall, the cellulose solubility in tetraalkylammonium hydroxide with a ratio of 11.25:1 was generally higher than it was at

22.5:1, especially for $[N_{666}][OH]$ where 5 wt% cellulose could be dissolved. The results for $[N_{666}][OH]$ agrees with Abe *et al.*, who recently reported 0.5 wt% cellulose could be dissolved for $\leq 15H_2O$, but not with $\geq 20H_2O$.²⁰ The $[N_{1111}][OH]$ was the exception, with even 0.25 wt% cellulose being insoluble at a ratio of 11.25:1. Lieser reported that the ease of cellulose dissolution in tetraalkylammonium hydroxides increased with increasing molecular volume of the cation,⁵⁰ but did not investigate up to $[N_{4444}][OH]$ or beyond. The observed trend of the “Goldilock’s zone” (cation neither too large nor too small) was maintained over the different water contents. Recent work into why aqueous $[N_{4444}][OH]$ is an effective cellulose solvent have highlighted the amphiphilic nature of the cation,²⁴⁻²⁵ allowing it to solvate hydrophobic pockets that water and hydroxide cannot. This allows us to conclude that there is an optimum degree of hydrophobicity; the $[N_{3333}]^+$ is small enough and hydrophobic enough to effectively solvate cellulose, whereas $[N_{1111}]^+$ and $[N_{6666}]^+$ represent the extremes of being not hydrophobic enough or too hydrophobic, respectively. A similar but weaker trend is also apparent for lignin solubility.

These preliminary results indicate that silica and lignin are highly soluble in the various hydroxides, with minimal cation effects, whereas cellulose is only highly soluble in the presence of $[N_{3333}]^+$ or $[N_{4444}]^+$ cations, with more limited solubility using $[N_{2222}]^+$ or $[N_{6666}]^+$. Solutions composed of $[N_{3333}][OH]$ or $[N_{4444}][OH]$ with a $H_2O:[OH]^-$ ratio of 22.5 or lower might therefore be predicted to be the most effective at whole rice husk disruption.

Enzymatic hydrolysis of rice husk pre-treated by hydroxides

Whole rice husks were ‘pre-treated’ with $[cation][OH] \cdot 22.5H_2O$ using a 2 wt% loading of rice husks. These were stirred in the hydroxide solutions at room temperature for 72 h. The

extended duration allowed evaluation of cation effects upon the overall pre-treatment effectiveness, separate from kinetic aspects (which were investigated later). Following addition of a suitable anti-solvent, solids were recovered, analysed and subjected to enzymatic hydrolysis (full details in Experimental Section).

Both methanol and water were investigated as anti-solvents. Methanol encouraged aggregation of the residue and solid-liquid separation (via filtration) was rapid and facile. When water was employed as an anti-solvent the filtration was significantly slower, but the use of water is more desirable from a sustainability perspective. The glucose yields from the pre-treated material are summarised in Figure 2.

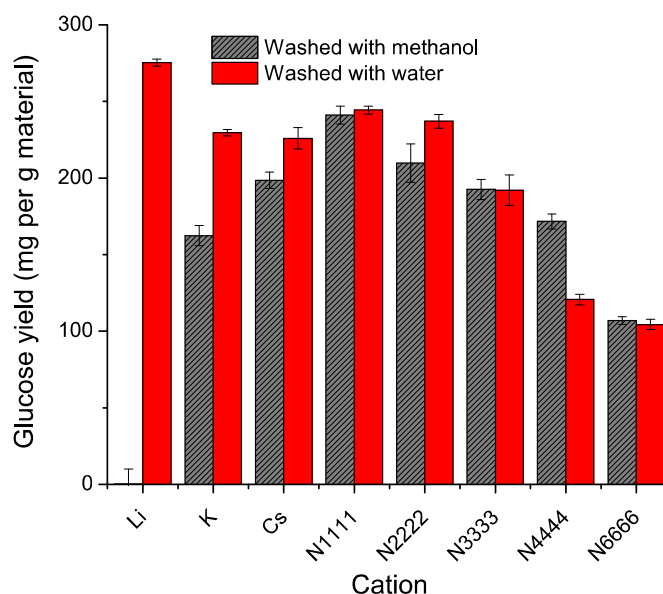


Figure 2: Enzymatic hydrolysis yields obtained from rice husks after pre-treatment by hydroxide solutions (all $\text{H}_2\text{O} : [\text{OH}^-] = 22.5 : 1$), as a function of both cation (x-axis) and either water (red) or methanol (shaded gray) as anti-solvent and washing solvent. No pre-treatment of the rice husks resulted in zero (or undetectable) glucose release upon enzymatic hydrolysis.

Briefly, for the rice husks pre-treated with alkali metal hydroxides; (i) washing with methanol was less effective than washing with water; (ii) if washing with water, lithium was the most effective of all cations. Conversely, pre-treatment with tetraalkylammonium hydroxides lead to the trends; (iii) as the cation size increased (from $[N_{1111}]^+$ to $[N_{6666}]^+$), the glucose yield dropped, (iv) the glucose yield was insensitive to whether the pre-treated rice husks were washed with water or methanol prior to enzymatic hydrolysis.

The water vs methanol difference can be easily rationalised by solubility differences. The tetraalkylammonium hydroxides investigated are highly soluble in both water and methanol, whereas the alkali metal hydroxides are sparingly soluble in methanol, with lithium hydroxide being the least soluble. Evaluation of the enzymatic broth confirmed that when methanol washing was employed, sufficient quantities of alkali metal hydroxide were transferred into the broth to change the pH and inhibit the activity of the cellulase enzymes.

While the exact hydrated cation radii are unknown in our investigated systems, the radii for more dilute aqueous systems have been compiled.⁵⁴ These radius values are summarised in Table S1, and the glucose yield plotted against the radii in Figure S3; this shows a clear trend with the optimal radius corresponding to that of lithium, with larger or smaller hydrated cation radii corresponding to lower glucose yields.

In order to investigate why the enzymatic hydrolysis yield decreased with increasing cation size from $[N_{1111}]^+$ to $[N_{4444}]^+$ (*i.e.* the opposite trend to that of cellulose solubility), further investigation of the bulk and chemical composition of the pre-treated biomass was performed.

Effect of pre-treatment by hydroxides upon the bulk structure of the rice husks

Generally, biomass pre-treatment studies employ drying, grinding and sieving steps. However, this is extremely energy-intensive, and therefore this study has investigated whole (as-supplied) rice husks, in order to see if these steps can be by-passed. In order to investigate the effect of pre-treatment upon the bulk structure, the *ca.* 8 mm by 2 mm cylindrical rice husks were pre-treated (as described above) then filtered using a pore size of 0.56 mm. Anything that did not pass through the filter was classed as 'bulk residue'.

Figure S4 plots the proportion of 'bulk residue' vs the $\text{H}_2\text{O}:[\text{OH}]^-$ ratio for KOH, $[\text{N}_{1111}][\text{OH}]$ and $[\text{N}_{4444}][\text{OH}]$. At high water ratios, the three are essentially indistinguishable. The filter retained the majority of the rice husk with only removal of external silica and some lignin being observed. However, by a $\text{H}_2\text{O}:[\text{OH}]^-$ ratio of 22.5, both KOH and $[\text{N}_{1111}][\text{OH}]$ solutions resulted in 100% of the pre-treated material passing through the filter (*e.g.* the bulk structure digested into a fine powder), whereas the cellulose-dissolving $[\text{N}_{4444}][\text{OH}]$ left 60 wt% of the rice husk as 'bulk residue', which was gelatinous in nature. Significantly, going from the composition unable to dissolve cellulose ($\text{H}_2\text{O}:[\text{N}_{4444}][\text{OH}]^-$ ratio of 45 : 1) to compositions able to dissolve cellulose ($\text{H}_2\text{O}:[\text{N}_{4444}][\text{OH}]^-$ ratio's of 22.5 : 1 and 11.25 : 1) actually increased the quantity of bulk, residual material.

Undissolved material in the filtrate was centrifuged and washed to obtain a 'digested residue'. Figure S5 displays photographs of the obtained 'bulk residue' and 'digested residue' as a function of KOH: H_2O ratio, while Figure 3 displays microscope images of select systems. The raw rice husk displayed the typical grid pattern (Figure 3(a)), and after pre-treatment with $\text{H}_2\text{O}:[\text{OH}]^- = 45$, the middle was extensively disrupted and generally absent

while the denser tips (pedicel and awn⁵) were thinned and cracked but otherwise largely intact (Figure 3(b)). After treatment with $\text{H}_2\text{O}:[\text{OH}]^- = 11.25$, no parts larger than 0.56 mm were present (*i.e.* zero 'bulk residue') and microscopic evaluation of the 'digested residue' (Figure 3(c)) confirmed it was composed entirely of an extremely fine mixture of undissolved holocellulosic material.

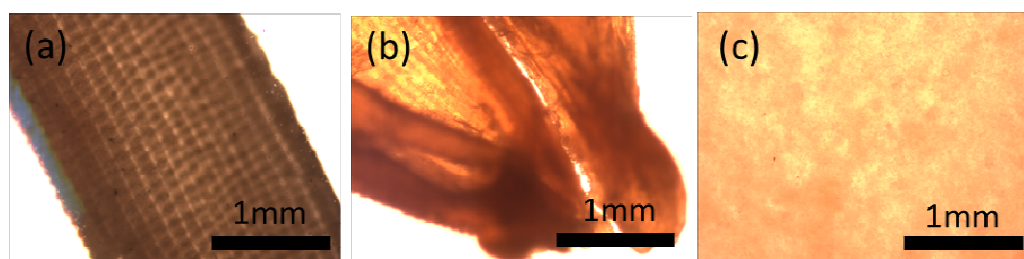


Figure 3: Microscope images of (a) the middle of an un-treated rice husk; (b) the edge of a rice husk pre-treated with relatively dilute KOH ($[\text{OH}]^- : \text{H}_2\text{O} = 45 : 1$), after being isolated as bulk residue and highlighting significant thinning and cracking; and (c) centrifuge residue after pre-treatment with relatively concentrated KOH ($[\text{OH}]^- : \text{H}_2\text{O} = 11.25 : 1$), highlighting how only a micron-sized fine suspension of the rice husk remains.

Significantly, the prior solubility tests indicated that $[\text{N}_{444}][\text{OH}]$ should have been able to form a true solution from all of the major constituent parts of the rice husk. However, the lignocellulosic components are held together within the biomass by various interactions (including covalent), and notably the cellulose present in biomass is at least an order of magnitude longer than Avicell cellulose. Ohno *et al.* have reported Avicell cellulose dissolution in minutes with aqueous hydroxide solutions,⁵⁵ but only complete dissolution of

whole biomass after a number of months;¹⁰ only 37% of polysaccharides could be removed by 60 wt% [P₄₄₄₄][OH] on the hour timescale,¹⁰ similar to what has been observed here. These differences can likely be attributed to the dramatic difference between isolated Avicell cellulose and intact cellulose in biomass. When Whatman filter paper no. 1 (*i.e.* composed of significantly longer cellulose chains⁴⁵) was added to [N₄₄₄₄][OH], dissolution was much slower and very significant viscosity increases (> 1,000 cP for 3 wt% filter paper cellulose) were observed.

Disintegration of the bulk lignocellulosic structure primarily requires lignin and hemicellulose removal. It is assumed that significant cellulose-[N₄₄₄₄][OH] interactions resulted in less effective disruption of the silica, lignin and hemi-cellulose bulk structure, as well as viscosity increases due to cellulose dissolution; this directly resulted in a reduced ability to disintegrate the bulk structure. The KOH and [N₁₁₁₁][OH] were only able to solvate the non-cellulosic components, and therefore rice husk structure disruption was much more significant. This more significant bulk-structure disruption qualitatively correlates with the higher glucose yield obtained from enzymatic hydrolysis of pre-treated rice husk.

Extent of silica and lignin removal

The lignin and silica present in the rice husks prevent enzymatic degradation of the cellulose by acting as a physical barrier, and by physisorbing the enzymes.^{8-9, 56-57} Silica is the main component on the rice husk surface, acting as an external armour and blocking the other components from reacting with the external environment.⁵⁷

Evaluation of the lignin and silica content of the bulk residue was hindered by the lack of bulk residue, although it was determined that what little bulk residue remained was essentially silica-free for $\text{H}_2\text{O}:[\text{cation}][\text{OH}]$ ratios of 22.5:1 or below. Addition of methanol as an antisolvent was therefore employed, followed by stirring for 20 min then filtration through a 10 μm pore filter. It was found that all $[\text{cation}][\text{OH}]\bullet 22.5\text{H}_2\text{O}$ solutions (followed by methanol addition) resulted in recovery of $65 \pm 5\%$ of the initial biomass (by dry weight), from which $60 \pm 10\%$ of the initial silica and $42 \pm 5\%$ of the initial lignin was removed. These results are consistent with the prior observations that alkali lignin and silica gel solubility demonstrates no significant cation dependency in the $[\text{cation}][\text{OH}]\bullet 22.5\text{H}_2\text{O}$ solutions. The process is therefore dominated by the chemical reaction of $[\text{OH}]^-$ with the solutes, *e.g.* to form $[\text{Si}(\text{OH})_4]^-$ and phenolates.

Rate of lignin extraction

All rice husk pre-treatment was performed for 72 h in order to exclude kinetic effects. However, it was noted that different $[\text{cation}][\text{OH}]$ solutions darkened at different rates. Therefore, the kinetics of lignin extraction over the first 24 h was evaluated by stirring 0.25 wt% rice husks in a cuvette and monitoring *in situ* using UV-Vis spectroscopy. Figure 4(a) displays typical spectra recorded for this process, demonstrating the growth of the expected lignin features at 300 nm and 334 nm. Figure 4(b) displays the raw hour-by-hour increase in absorbance at 334 nm for all of the different $[\text{cation}][\text{OH}]\bullet 45\text{H}_2\text{O}$ solutions; extraction was even faster in $[\text{cation}][\text{OH}]\bullet 22.5\text{H}_2\text{O}$ solutions.

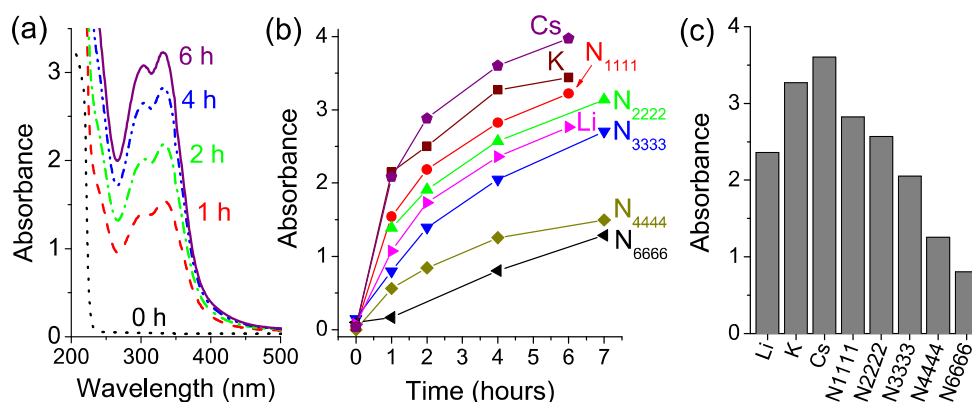


Figure 4: (a) UV-Vis spectra over time for 0.25 wt% rice husk stirred in a 10 mm cuvette with [N₁₁₁₁][OH] (H₂O : [OH]⁻ = 45 : 1), highlighting the growth of characteristic lignin absorption features at ca. 305 nm and 334 nm. (b) Raw data highlighting differing rates of lignin extraction from rice husk pre-treated by hydroxide solutions with a range of cations (all H₂O : OH = 45 : 1), plotting the absorbance at 334 nm vs time. (c) A summary of the data in (b), plotting absorbance at 334 nm at 4 hours vs the cation used, to highlight the trend in the significant cation effect upon the rate of lignin extraction.

Figure 4(c) summarises the cation trend observable in Figure 4(b), as a function of cation. Table S1 summarises both these absorbance values and the reported radius of the hydrated cations. These have been plotted in Figure S6, and a clear linear trend can be observed according to the size of cation, with a smaller hydrated radius correlating with more rapid lignin removal. There is also a qualitative similarity between the trend in Figure 2 (glucose yield after pre-treatment) and Figure 4(c) (rate of lignin extraction), although Li[OH] is a notable outlier here.

Evaluation of factors that can affect cellulase hydrolysis

The above results demonstrate relatively similar lignin and silica removal processes (as hydroxide-dominated processes) but cation-dependant destruction of the macro-structure, and lignin extraction kinetics. Possible cation-dependant effects upon the downstream enzymatic hydrolysis process were also investigated.

The hydrolysis of amorphous cellulose was investigated using cellulase in *ca.* pH 4.8, 50 mM [cation][acetate]/acetic acid buffers, to explore how residual cations from the pre-treatment might influence the activity of the enzymes. The expected amount of glucose (*ca.* 1,000 mg glucose per gram of cellulose) was eventually released for all systems, with the exception of $[N_{666}]^+$, which completely inhibited the cellulase enzymes. For the other cations, small inhibitory effects were observed upon the rate of hydrolysis; this is demonstrated by Figure 5(a), which shows the glucose yield after 24 h enzymatic hydrolysis, as a function of cation. The degree of inhibition increased in line with the Hofmeister's series⁵⁸ and the trend from kosmotropic to chaotropic systems. However, this means that except for $[N_{666}]^+$, residual cations cannot have influenced the observed glucose yields from pre-treated rice husks, since the reaction was allowed to go to completion (over 48 - 72 h).

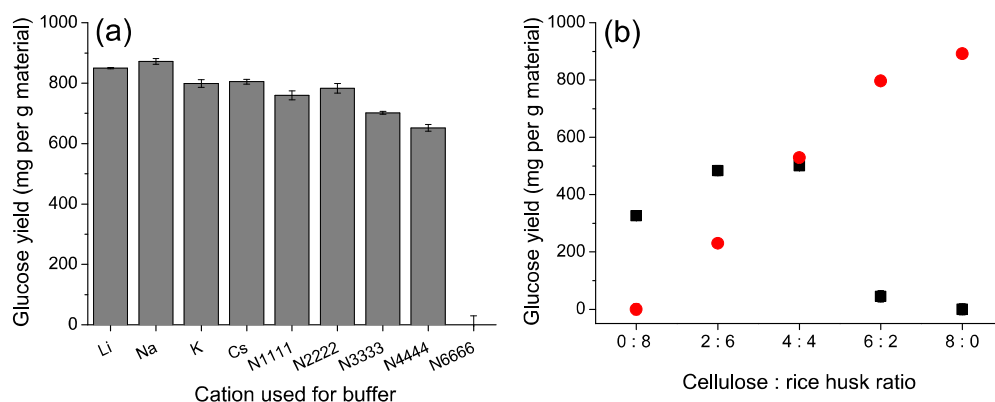


Figure 5(a): Glucose yield after 24 h enzymatic hydrolysis of filter paper cellulose with cellulase in ca. pH 4.8, 50 mM [cation][acetate]/acetic acid buffers, as a function of cation, highlighting some enzyme inhibition for the larger cations. All reached the expected value of 1,000 mg per g after 72 h, except the $[N_{666}]^+$ -based buffer; and **(b)** glucose yield after 48 h enzymatic hydrolysis, for a range of rice husk and cellulose mixtures, either (●) untreated or (■) pre-treated by $[N_{444}][OH] \cdot 22.5H_2O$. The total loading was fixed at 2.5 wt%, and highlights that pre-treatment was moderately beneficial for the lignocellulosic biomass but increasingly detrimental as the proportion of cellulose in the system increased.

The pH is an extremely influential parameter for cellulase enzymes, with a pH of 4.8 typically optimal.⁵⁹ Therefore, studies were performed as a function of cellulose content, degree of washing post-treatment, and the pH during extended hydrolysis.

Firstly, the enzymatic hydrolysis of cellulose:rice husks in a ratio of 0:8, 2:6, 4:4, 6:2, 8:0 were investigated with pre-treatment in $[N_{444}][OH] \cdot 22.5H_2O$. The glucose yield significantly increased for pre-treatment of the rice husk, but dropped to zero for pure cellulose (Figure 5(b)). This was found to be due to significant changes in the pH of the

enzymatic broth, which was largely unchanged for pre-treated and rinsed rice husks, but reached pH 12 for the pre-treated cellulose, despite an identical washing process. Therefore the cellulose-dissolving $[N_{4444}][OH] \cdot 22.5H_2O$ was significantly more difficult to remove from cellulose than from the lignocellulosic biomass.

Next, a detailed study investigated the effect of washing upon rice husks pre-treated with KOH, $[N_{1111}][OH]$ and $[N_{4444}][OH]$. Prior experiments using Na[OH] pre-treatment of rice straw have noted no washing³³ or very extensive washing²⁸ was required prior to cellulase treatment, highlighting some discrepancy. Solids recovered after pre-treatment of rice husks with [cation][OH]:22.5H₂O solutions and precipitation with methanol (10 times the original volume) were washed with water (1, 3 or 10 times the initial treatment solution volume) or used directly (*i.e.* no washing). The solid was then transferred to the enzymatic broth and the resulting pH of the solution recorded.

For KOH, no washing released virtually no glucose and corresponded to a high pH; one or more washes resulted in the expected pH of 4.8 and consistent glucose yields. These pH values are displayed graphically in Figure S7. For $[N_{1111}][OH]$ and $[N_{4444}][OH]$, increased washing volumes resulted in drops in pH and associated improvements in the glucose yield, but pH 4.8 was not achieved until after 10 washes, demonstrating the difficulty of removing these from the pre-treated biomass. Importantly, as the length of the alkyl chain increases, the surfactant-like nature of the cation increases, as does its hydrophobic interactions with the cellulose, accounting for the more difficult removal of these cations from the biomass and, by extension, the hydroxide anion. While acid-washing the cellulose could overcome this issue (as reported by others²⁸), this is significantly less desirable when considering possible recycling of used hydroxide solutions.

Finally, Avicell cellulose was dissolved in $[N_{4444}][OH] \cdot 22.5H_2O$, precipitated by water addition, thoroughly washed with methanol and water, and then transferred to the enzymatic hydrolysis broth. The pH of the broth was confirmed to be 4.8, whereas within 48 or 72 h (by which stage the solid cellulose had been completely deconstructed by the enzymes), the pH of the broth rose to between pH 5.3 and 5.8. This strongly indicates that some of the cellulose-dissolving $[N_{4444}][OH]$ is trapped inside the cellulose matrix during the rapid precipitation of the cellulose. The intercalation of $[P_{4444}]^+$ and $[N_{4444}]^+$ cations inside regenerated biomass has recently been demonstrated by Energy-dispersive X-ray spectroscopy⁵ and Solid-State NMR,⁶⁰ respectively. The encapsulated material can therefore be progressively released during enzymatic hydrolysis, potentially in sufficient quantities to inhibit further digestion of the cellulose material.

Discussion

As noted at the outset, the cation is a strong determining factor in the ability of water-deficient (or partially hydrated) hydroxide electrolytes to dissolve cellulose (*cf.* Figures 1, S2). The cation also has a significant effect upon biomass disruption (*cf.* Figures 3, S4, S5) and ultimately the enzymatic hydrolysis yield (*cf.* Figures 2, S3), but these positive outcomes are *inversely* correlated with an ability to dissolve biomass.

Figure 6 is a diagram highlighting the proposed pre-treatment of the rice husks on the microscopic and molecular scale. Silica and lignin removal are important processes but were essentially cation-independent (after an anti-solvent was added, and if kinetics are omitted) so are not considered in detail in the Figure. In Figure 6, process I is the disruption

and swelling of the cellulose microfibrils. Importantly, the degree of splitting of microfibrils by hydroxide solutions has been reported⁶¹ to have the similar cation trend of $[N_{1111}]^+ > Li^+ > Na^+ > K^+$ as is seen in this work for the enzymatic hydrolysis results. Process II is the insertion of the cation into the cellulose lattice, swelling the cellulose microfibrils and potentially converting crystalline cellulose to amorphous cellulose; there is discrepancy in the literature as to whether $Li[OH]$ or $Na[OH]$ is a more effective swelling agent,⁶² but $[N_{1111}][OH]$ is generally recognised as superior to $Na[OH]$.^{22-23, 46} Processes I and II are also linked, since it is uneven rates and extents of process II that results in process I occurring.⁶¹ Finally, some of the cations are able to achieve cellulose dissolution (Process III). Only systems such as $[N_{4444}][OH]$ are able to perform all three processes. However, process III is the most intensive in terms of ion pairs required (potentially up to an ion pair per cellulosic hydroxyl group) and therefore dissipates a significant quantity of the chemical potential inherent in these hydroxide electrolytes. Conversely, the chemical potential of solutions such as $Li[OH]$ and $[N_{1111}][OH]$ are utilised in effectively performing Processes I and II, as well as disrupting the bulk structure of the biomass. Solutions such as $Li[OH]$ and $[N_{1111}][OH]$ are also easily removed from the pre-treated biomass by rinsing, whereas the more significant (hydrophobic-hydrophobic) interactions and intimate association between $[N_{4444}][OH]$ and cellulose results in $[N_{4444}][OH]$ -incorporation inside the pre-treated biomass.

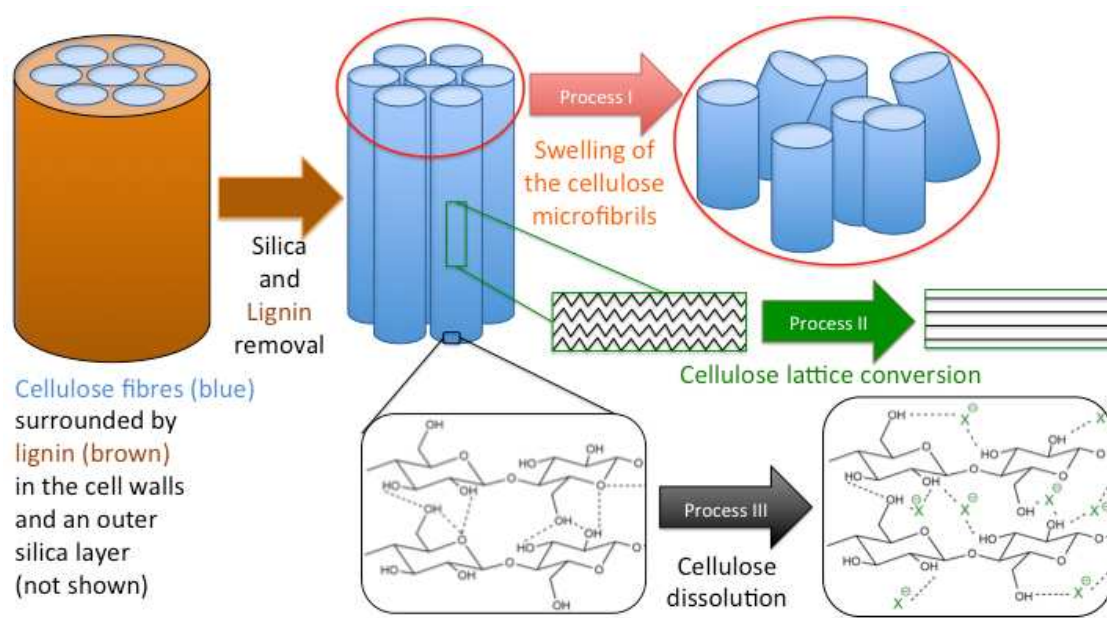


Figure 6: Schematic diagram highlighting the three main processes cellulose macro/microfibrils can undergo upon alkaline hydroxide treatment; process I is splitting of the macrofibrils into microfibrils, process II is swelling and lattice conversion of the microfibrils, and process III is molecular dissolution.

There appears to be a clear size and hydrophobicity trend in terms of effectiveness at whole biomass pre-treatment, e.g. $[N_{666}][OH]$ is too hydrophobic so interacts relatively weakly with all components beyond chemical reaction with silica. Systems such as $[N_{444}][OH]$ are hydrophobic enough to enable extensive interactions with cellulose, but to the detriment of pre-treatment of the whole. Small cations appear to demonstrate more rapid lignin extraction from the biomass (cf. Figure S6), but if left to reach equilibrium then the extent of lignin removal is comparable. Other processes such as cellulose swelling have been related to the cation's ability to promote hydroxide access into cellulosic fibres, with $[N_{111}][OH]$ and $Li[OH]$ previously being identified as particularly effective;⁶¹ our results have

also shown $[N_{1111}][OH]$ and $Li[OH]$ to be particularly effective at pre-treatment of the whole biomass, when judged by enzymatic hydrolysis of the resulting cellulose.

All strongly alkaline solutions are hazardous to human health, by virtue of their corrosive nature.³⁷ Considering possible downstream fates of the cations, alkali metal cations are omnipresent in the environment. Conversely, tetrabutylammonium is foreign to the environment and there is no evidence that it undergoes any biodegradation under standard conditions.⁶³ The tetramethylammonium cation is endemic to nature, but is also a ganglionic blocker, making tetramethylammonium hydroxide lethally toxic to humans in high enough doses.³⁷ Given the relatively greater abundance, relatively lower toxicity, sustainability and economic advantages inherent in the alkali metal systems, these appear to be much more viable pre-treatment media than tetralkylammonium-based hydroxide systems. An ability to dissolve cellulose is actually detrimental (in the conditions employed in this study). However, the complete dissolution of biomass is still desirable for alternative goals, such as the complete extraction of value-added molecules from a lignocellulosic matrix.⁶⁰

Conclusions

Lignocellulosic biomass is extremely robust against enzymatic attack, such as hydrolysis in a cellulase enzymatic broth to form glucose; pre-treatment is required to facilitate this enzymatic process. After investigating eight different $[cation][OH]$ systems, we have identified a significant cation effect upon pre-treatment effectiveness (as judged by enzymatic hydrolysis). Four of the eight solutions were able to dissolve significant quantities of cellulose, but this ability was generally detrimental with respect to the final glucose yield.

The tetraalkylammonium hydroxide systems that were able to dissolve cellulose have 'surface active' (surfactant-like) cations, which were relatively difficult to remove from the pre-treated biomass surface. In particular, the tetraalkylammonium hydroxide systems most effective at dissolving cellulose were virtually impossible to remove from the cellulose, given that they are trapped inside the treated biomass when solvent conditions are changed. Therefore, when biomass disruption is intended, minimal washing is desired and enzymatic hydrolysis is the end goal, cellulose-dissolving hydroxide solutions are *not* appropriate. Lithium and tetramethylammonium hydroxide were most effective, but the latter is highly toxic. This allows us to conclude that in such situations, conventional alkali metal hydroxide solutions such as lithium hydroxide are expected to be superior in terms of effectiveness as well as cost, availability, toxicity and stability.

Acknowledgements

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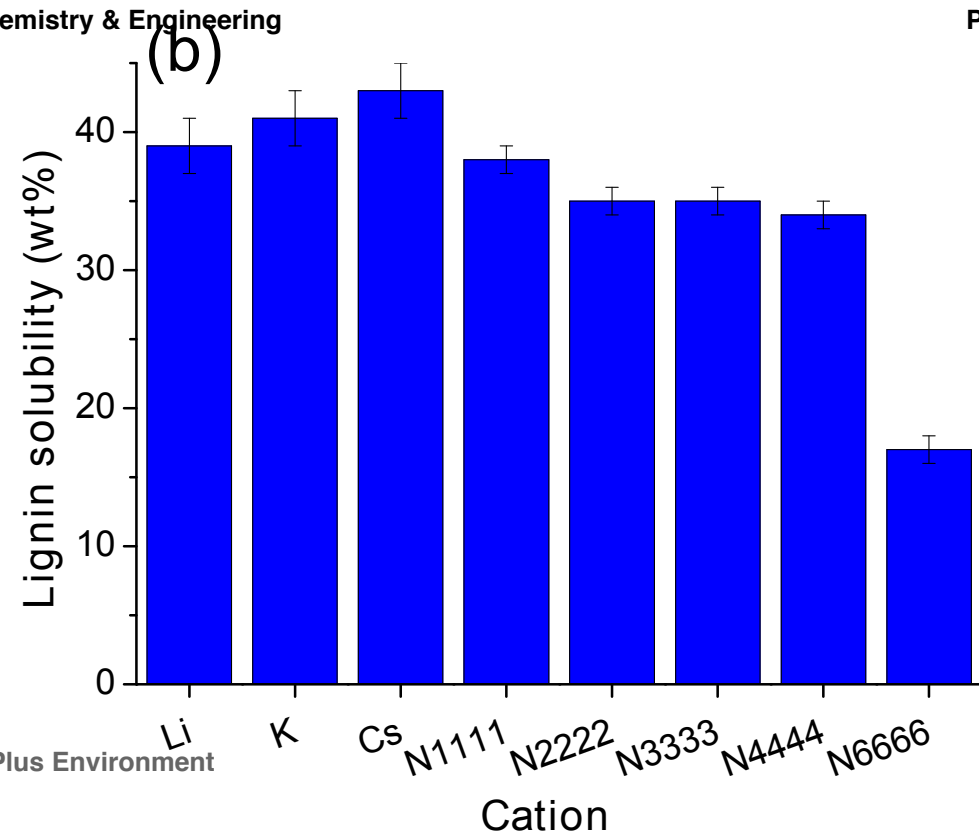
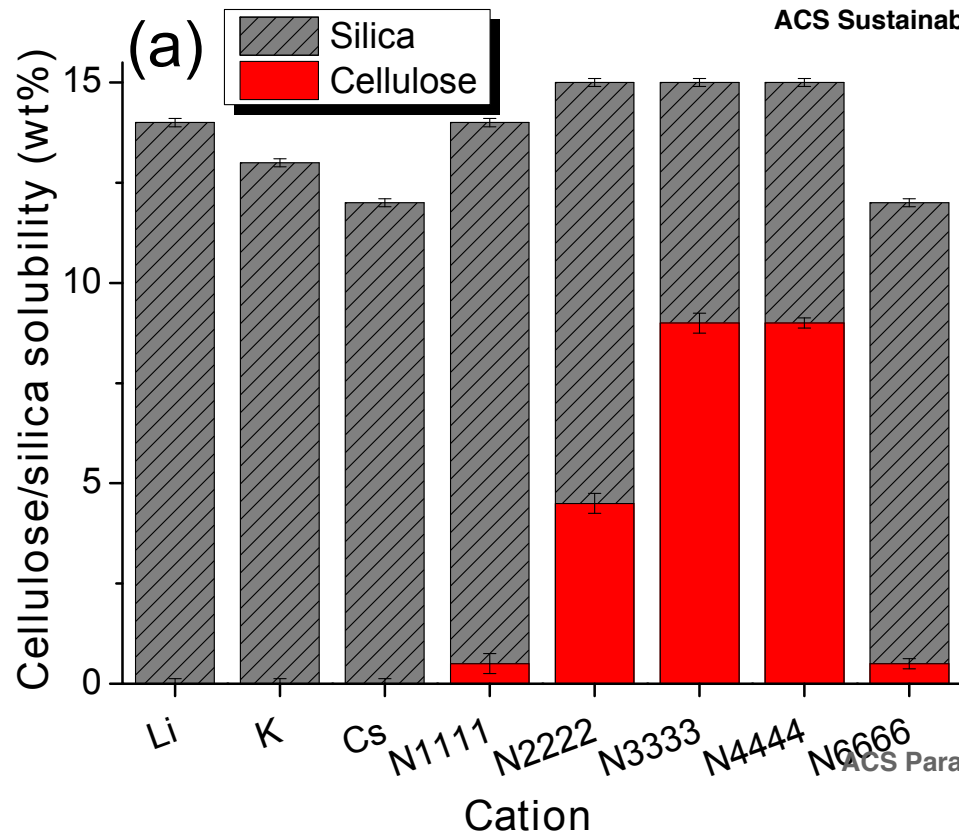
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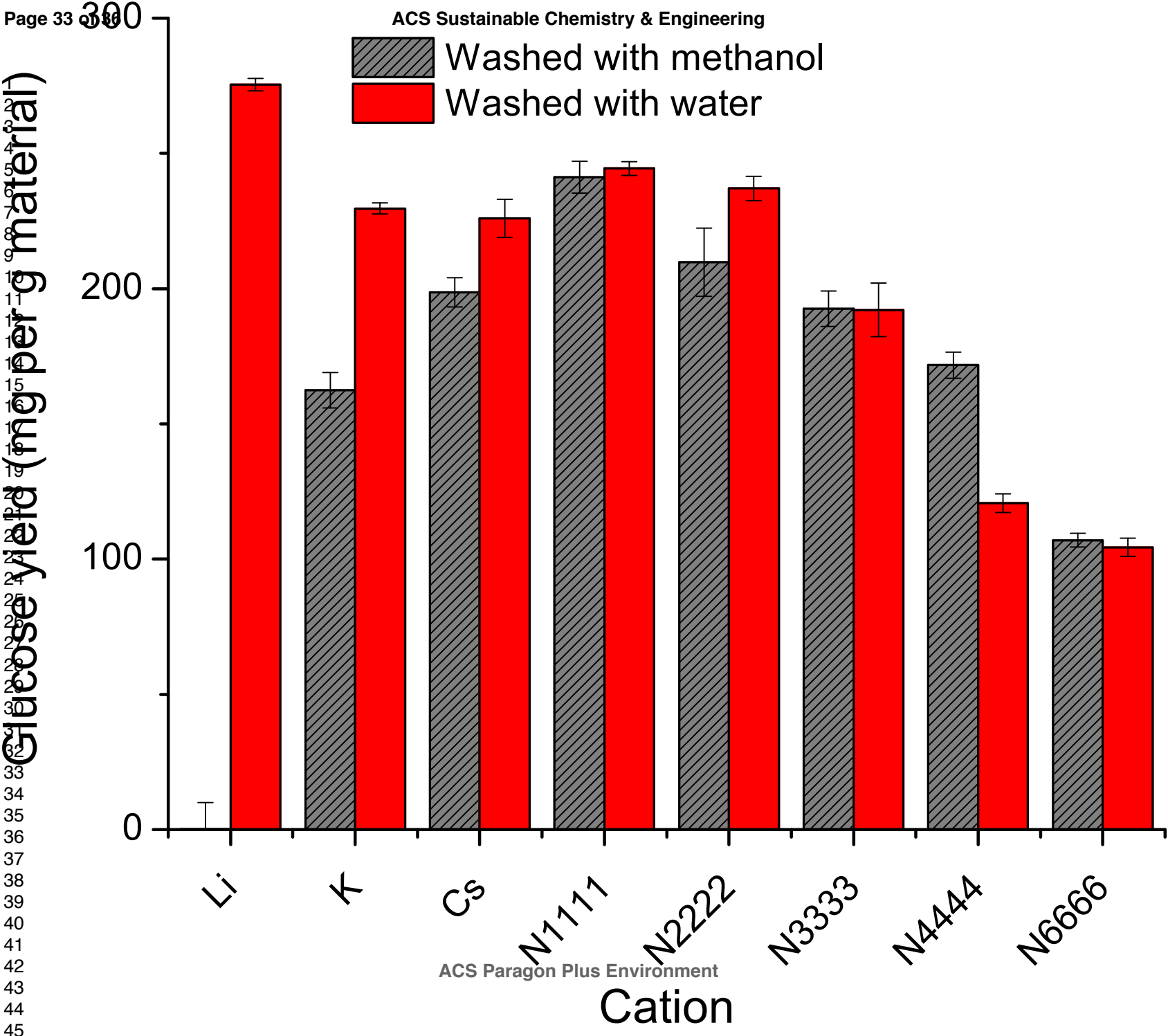
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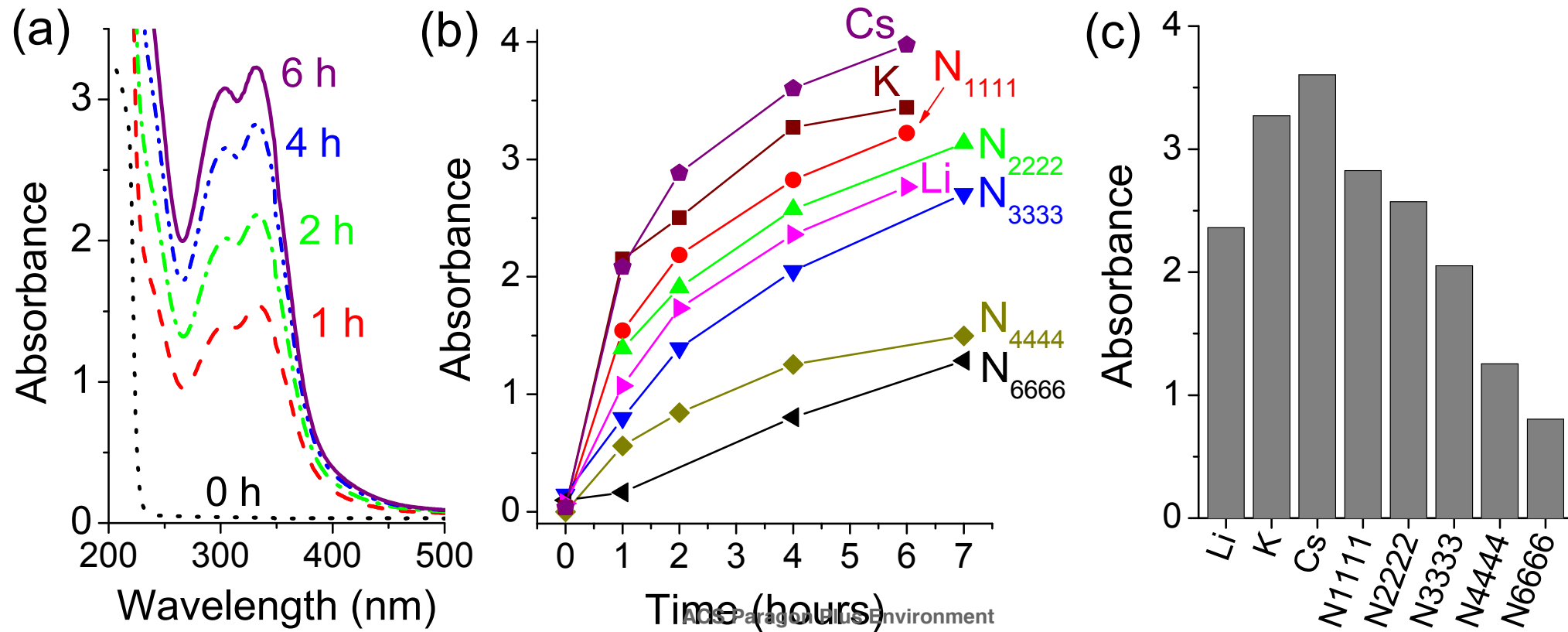
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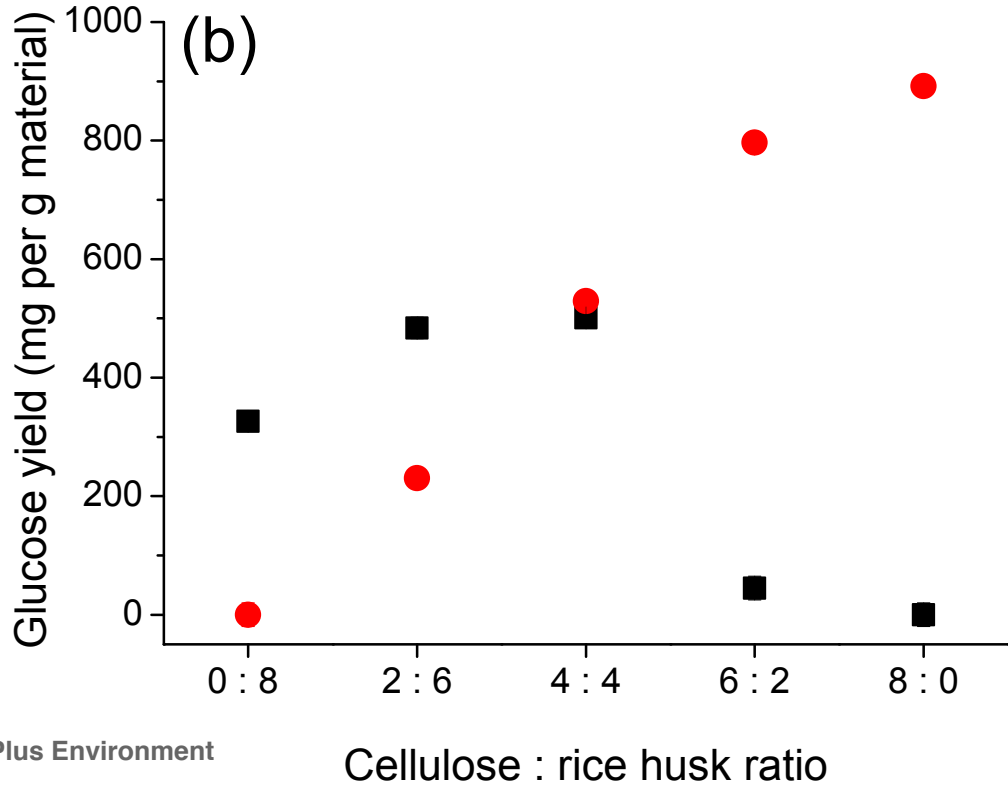
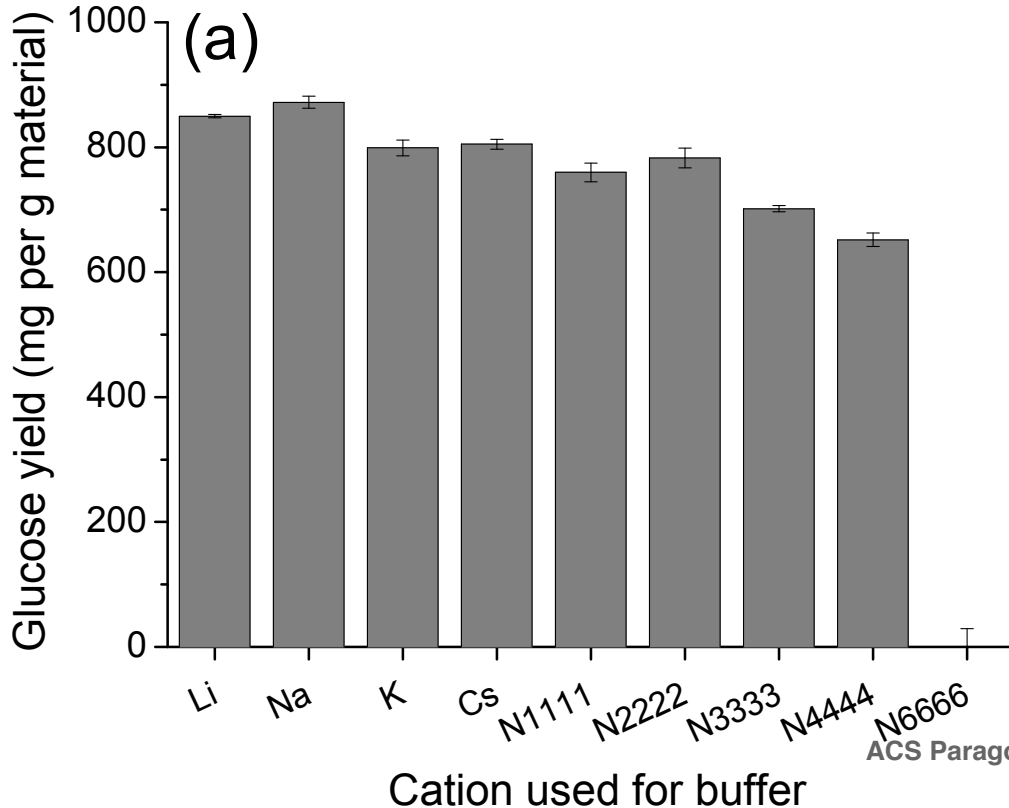


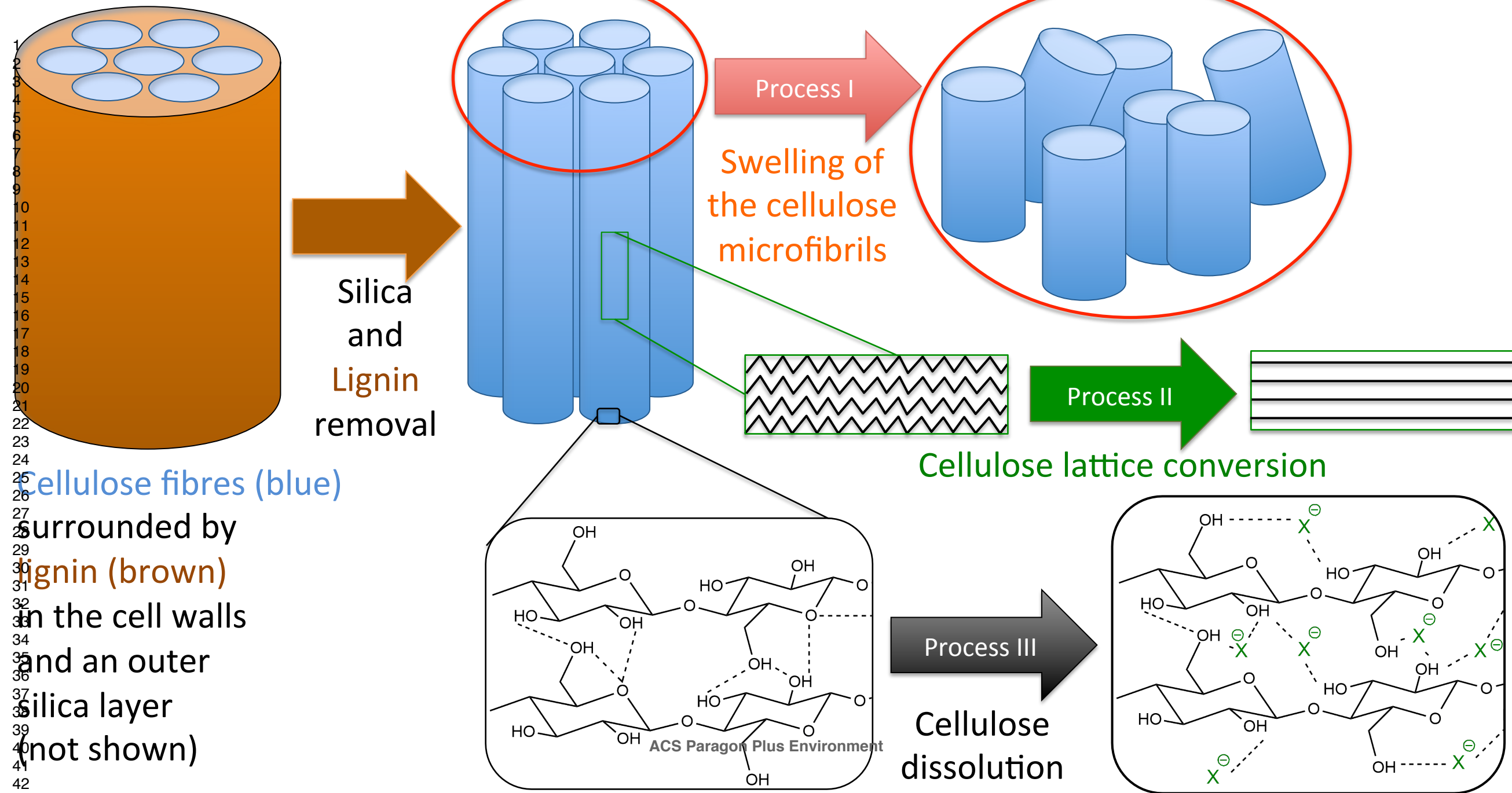
Glucose yield (mg per g material)

33
34
35
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Electronic Supporting Information for

“A cation study on rice husk biomass pre-treatment with aqueous hydroxides: Cellulose solubility does not correlate with improved enzymatic hydrolysis”

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This Supporting Information contains 1 Table and 7 Figures, over 9 pages (pages S1 to S9).

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Table S1: A summary of the literature values^(a) reported for the hydrated cation radius of the various cations, and the outcomes when hydroxide solutions using these particular cations were used for pre-treatment prior to enzymatic hydrolysis^(b) and lignin extraction^(c). These values were used to plot Figures S3 and S6.

Cation	Reported hydrated cation radius / pm ^(a)	Glucose yield / mg per g rice husk ^(b)	Absorbance from lignin extraction ^(c)
Li ⁺	382	275	2.36
K ⁺	331	230	3.27
Cs ⁺	329	226	3.60
[N ₁₁₁₁] ⁺	347	244	2.83
[N ₂₂₂₂] ⁺	400	237	2.57
[N ₃₃₃₃] ⁺	452	192	2.05
[N ₄₄₄₄] ⁺	494	121	1.25
[N ₆₆₆₆] ⁺	566 ^(a)	104	0.81

^(a) Li⁺ to [N₄₄₄₄]⁺ values come from compiled values reported in Volkov, A. G.; Paula, S.; Deamer, D. W., Two mechanisms of permeation of small neutral molecules and hydrated ions across phospholipid bilayers. *Bioelectrochemistry and Bioenergetics* **1997**, 42 (2), 153-160. The value for [N₆₆₆₆]⁺ was estimated by fitting the radius for [N₁₁₁₁]⁺ to [N₄₄₄₄]⁺ vs the number of carbons in the alkane chain, fitting a 2nd order polynomial curve ($R^2 = 0.999$) and extrapolating to the hexyl analogue.

^(b) Glucose yields after 48 h enzymatic hydrolysis, for rice husks pre-treated in [cation]OH (all H₂O : [OH]⁻ = 22.5 : 1), after use of water as the anti-solvent.

^(c) Absorbance recorded at 334 nm after stirring 0.25 wt% rice husk for 4 h in [cation][OH] solutions (H₂O : [OH]⁻ = 45 : 1).

Figure S1: Solubility of silica gel (in wt%) in aqueous solutions of K[OH], [N₁₁₁₁][OH] and [N₄₄₄₄][OH], as a function of water content. The results demonstrate that increasing the hydroxide content increases silica solubility (likely by reaction to form the solvated silicate anion), up to a maximum solubility at [cation][OH]•22.5H₂O; further removal of water slightly decreases silica solubility, likely due to the key role of water in solvating the silicate anions. Only a relatively minor cation effect can be observed.

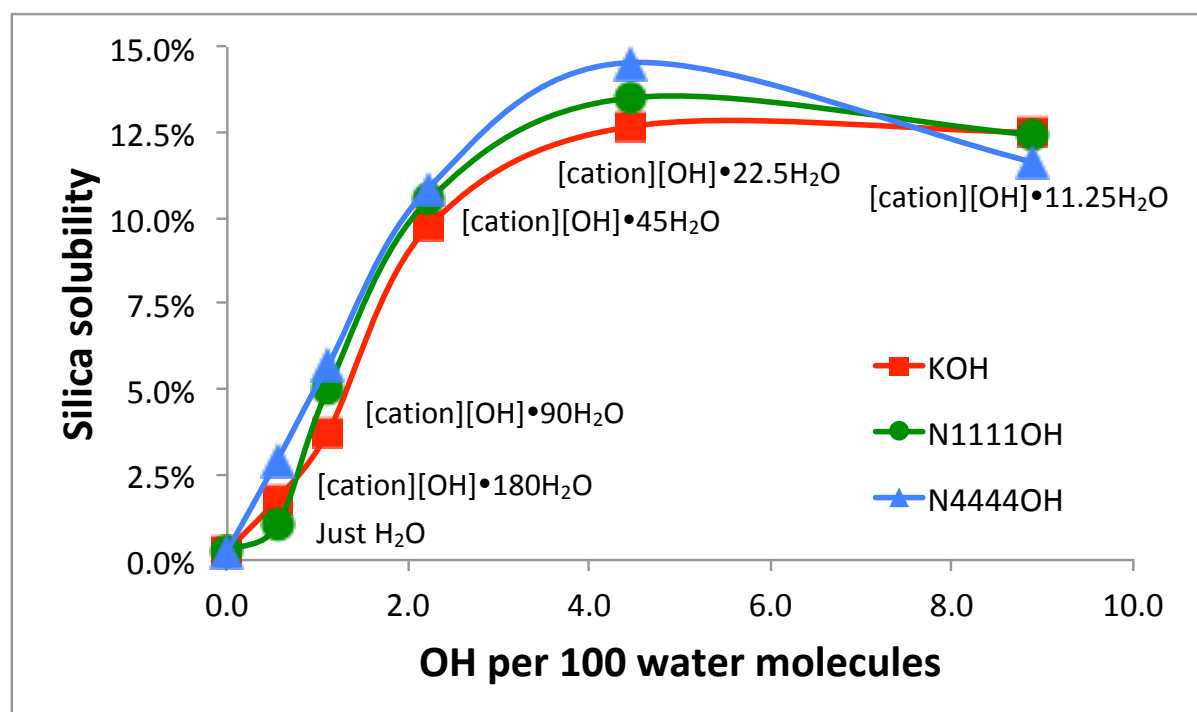


Figure S2: Room temperature solubility of microcrystalline cellulose in [cation][OH]•xH₂O, as a function of cation and for three H₂O:[OH][−] ratios (x = 11.25, 22.5 or 45), highlighting both a significant cation size effect and a water content effect upon cellulose solubility. All systems with solubility exceeding 5 wt% eventually became too viscous (typically gelled) to stir, rather than reaching a point of genuine saturation; systems with lower solubility values presented a transparent solution with undissolved cellulose (present as a powder) at the point of saturation. Please note that Li[OH]•11.25H₂O could not be physically prepared for solubility reasons.

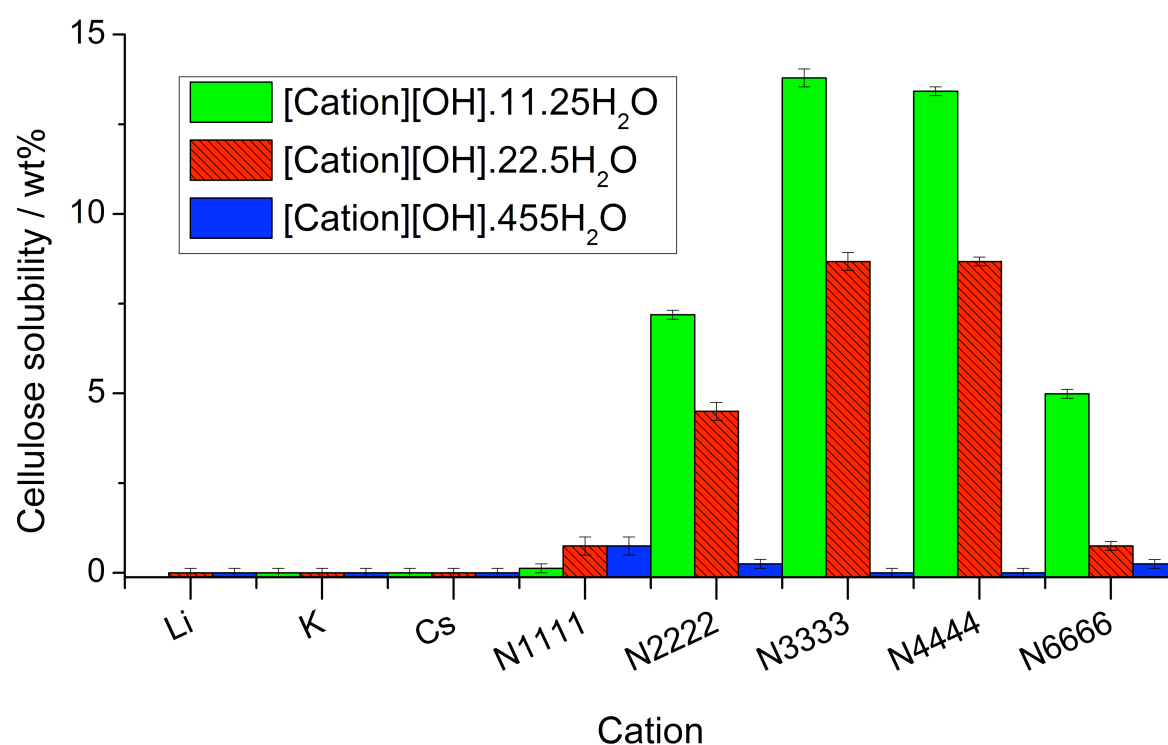


Figure S3: Chart re-plotting the glucose yield after enzymatic hydrolysis of rice husks pre-treated with $[\text{cation}][\text{OH}]\cdot 22.5\text{H}_2\text{O}$, and after water was used as anti-solvent (*cf.* Figure 2 in the manuscript) as a function of the reported hydrated cation radius (*cf.* Table S1). This appears to indicate a clear optimum size for the cation radius, although it must be emphasised that the hydration state of the cations in these relatively concentrated solutions are not yet known.

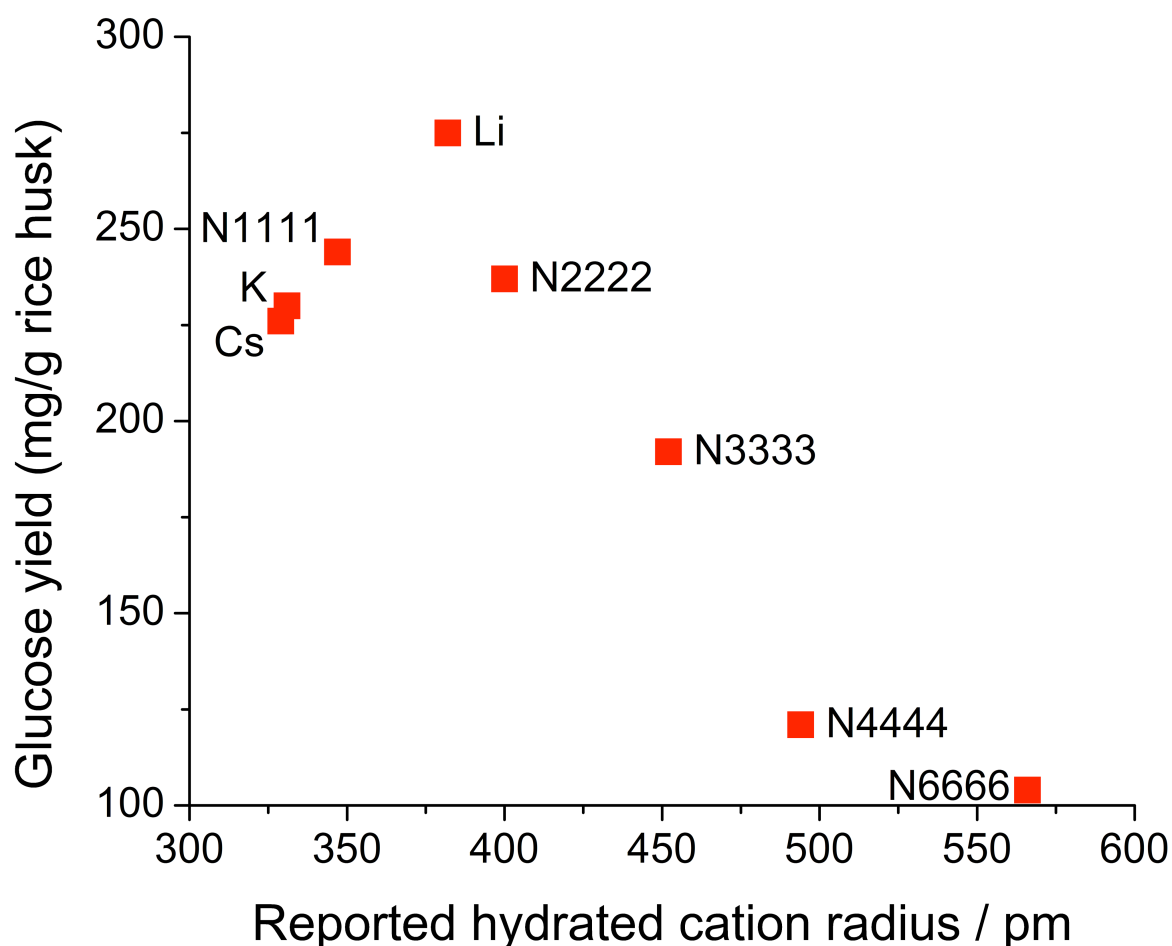


Figure S4: Chart plotting the mass of rice husks (ca. 8 mm by 2 mm cylinders) that passed through a 0.56 mm diameter hole when filtered, after being stirred for 72 h in [cation][OH] \cdot xH₂O, as a function of the H₂O:[OH][−] ratio and cation. Simply stirring in water resulted some material passing through the filter, due to partial fragmentation of the rice husk during handling, stirring and filtering. As the quantity of [OH][−] increased, the amount of material passing through increased as the silica and lignin were dissolved. Concentrated solutions of K[OH] and [N₁₁₁₁][OH] (NB: still unable to dissolve cellulose) deconstructed the bulk rice husks into fragments all < 0.56 mm, so no bulk material was retained by the filter. Once [N₄₄₄₄][OH] was concentrated enough to dissolve cellulose, deconstruction of the rice husk was actually detrimentally effected, and more of the rice husk was retained by the filter as a gelatinous mass.

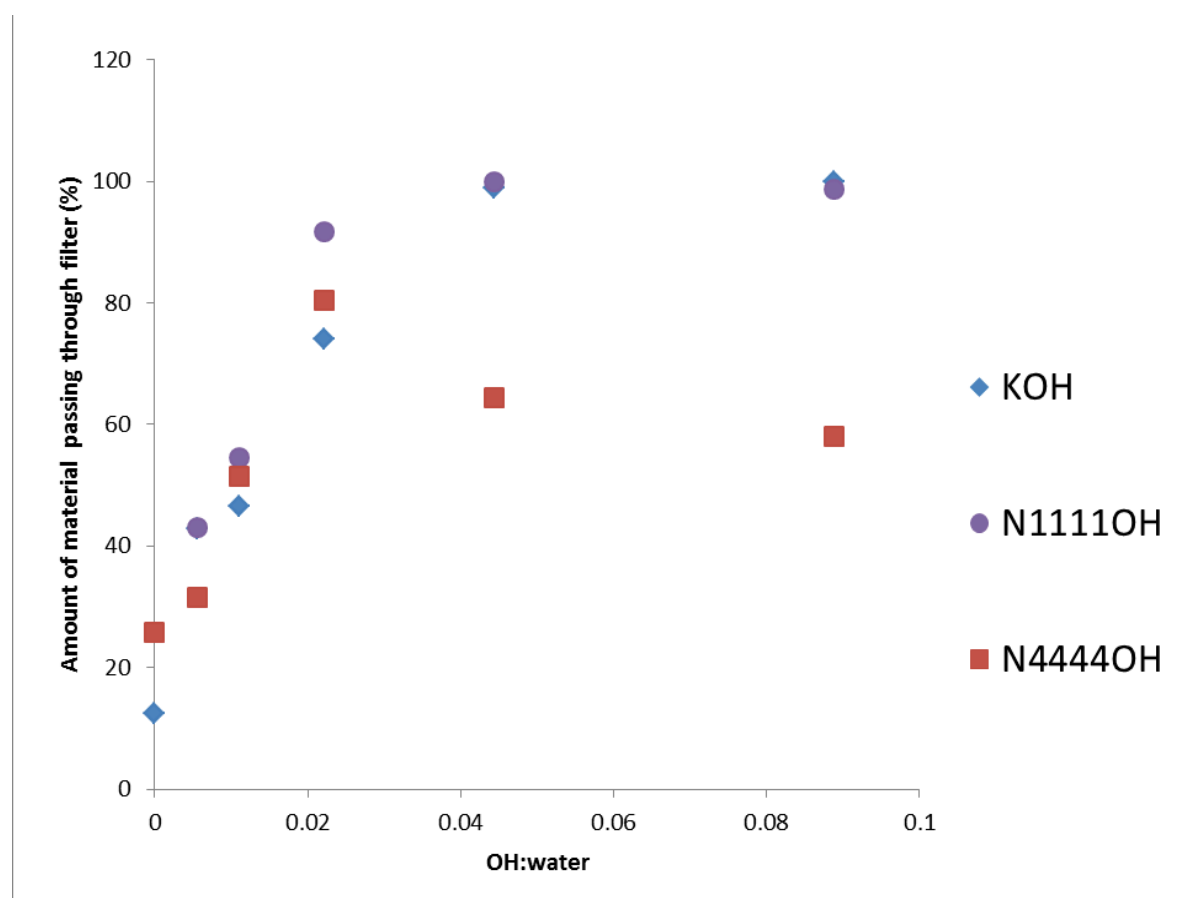


Figure S5: Photographs of (top) ‘bulk residue’ and (bottom) ‘centrifuge residue’ for five different $K[OH] \cdot xH_2O$ ratios, which are (from left to right) $x = 180, 90, 45, 22$ and 11.25 . These correspond visually to the numerical values presented in Figure S3. As can be clearly seen, as the quantity of water in the system decreased, the ability of the aqueous hydroxide system to deconstruct the rice husks increased. No rice husk pieces of significant size remained after pre-treatment, and the filtrate was composed of a solution of lignin and silica, and a suspension of undissolved, micron-sized holocellulose particles.



Figure S6: Chart plotting the absorbance representing lignin extraction shown in Figure 4(c) in the manuscript, as a function of the reported hydrated cation radius (*cf.* Table S1). This appears to indicate a clear linear correlation between the rate of lignin extraction from rice husks after 4 h vs the size for the hydrated cation in aqueous solution, with smaller cations resulting in more rapid lignin extraction. However, the precise hydration state of the cations in these relatively concentrated solutions are not yet known.

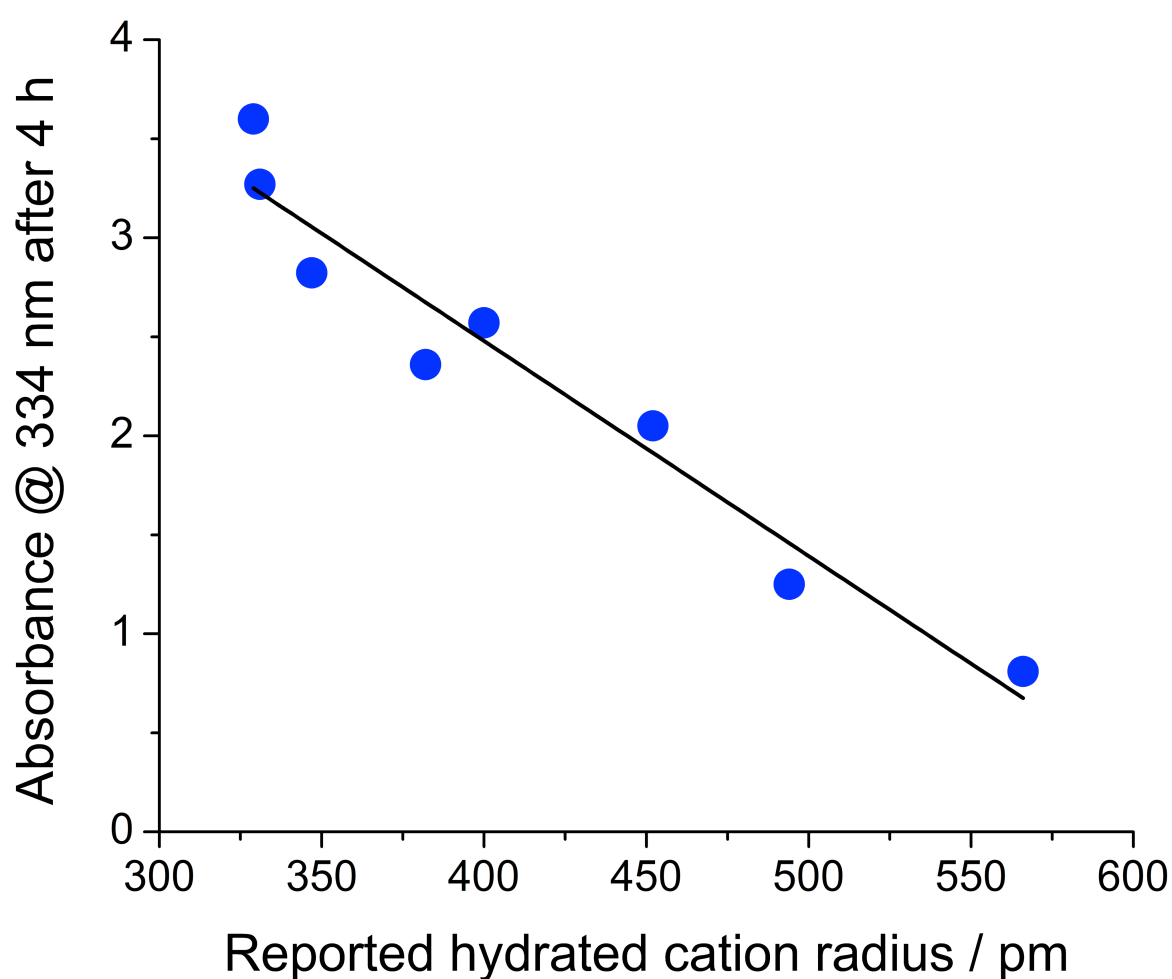


Figure S7: Plot representing the pH of an initially pH 4.8, 50 mM sodium acetate/sodium acetate, after the addition of pre-treated rice husks as a function of the pre-treatment solution ($\text{K}[\text{OH}] \cdot 22.5\text{H}_2\text{O}$, $[\text{N}_{1111}][\text{OH}] \cdot 22.5\text{H}_2\text{O}$ and $[\text{N}_{4444}][\text{OH}] \cdot 22.5\text{H}_2\text{O}$) and the number of washes prior to the isolated solid being added to the enzymatic broth. Zero washes indicate only addition of methanol as an anti-solvent (50 mL methanol for 5 mL aqueous hydroxide solution) and the solid was then collected by filtration and transferred directly. Washing indicates the number of equivalents of water added in 5 mL aliquots to the solid on the filter (e.g. 1 wash = one 5 mL aliquot of water). Very little ‘washing’ was required to remove the residual $\text{K}[\text{OH}]$ from the rice husks, whereas an order of magnitude more was required to remove a similar quantity of $[\text{N}_{4444}][\text{OH}]$.

